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(71) Applicant *(for GB only)*: **PFIZER LIMITED** [GB/GB]; Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

(71) Applicant *(for all designated States except GB, US)*: **PFIZER INC.** [US/US]; 235 East 42nd Street, New York, NY 10017 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GREENGRASS, Pamela, May** [GB/GB]; Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB). **STEWART, Michael** [GB/GB]; Pfizer Limited, U.K. Patent Department, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB). **WOOD, Claire, Margaret** [GB/GB]; Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

(74) Agents: **HAYLES, James, R.** et al.; Pfizer Limited, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

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(54) Title: ASSAY

(57) **Abstract:** The invention relates to an assay to establish the affinity of compounds at the "ether-a-go-go" (ERG) potassium (K+) channel, in particular the human ERG (hERG) potassium channel, using a labelled inwardly rectifying potassium channel (IKR) blocker. This assay is useful to identify compounds with undesirable effects on cardiac repolarisation in man, in particular the propensity to prolong the QT interval in the electrocardiogram.

Assay

The invention relates to an assay to establish the affinity of compounds at the "ether-a-go-go" (ERG) potassium (K^+) channel, in particular the human ERG (hERG) potassium channel, using a labelled rapid delayed rectifying potassium channel (IKR) blocker, for example [^{3}H]-dofetilide or [^{3}H]-MK-499. This assay is useful to identify compounds with undesirable effects on cardiac repolarisation in man, in particular the propensity to prolong the QT interval in the electrocardiogram, which may lead to Torsades de Pointes.

In recent years the development of some compounds proposed for therapeutic use has been abandoned in late phase drug development due to the detection of undesirable effects on cardiac repolarisation in man. The effects of these drugs are assessed in terms of the QT interval in the electrocardiogram (ECG). The QT interval is the portion of an ECG that represents the time from the beginning of ventricular depolarization to the end of ventricular repolarisation. Because the QT interval can be affected by heart rate lengthening with a decrease in heart rate and shortening with an increase in heart rate, the QT is often "corrected" for heart rate, resulting in the QT_C interval. In rare cases the administration of some drug molecules results in a prolongation of the QT interval of the ECG in man. The ECGs of these patients resemble those of individuals suffering from an inherited disorder known as long QT syndrome. Drug-induced ventricular fibrillation, in these cases, can eventually lead to sudden death (Morganroth J et al. (1993) Am J Cardiol. 72, 26B-31B; De Ponti F. et al., (2000) Eur J. Clin. Pharmacol. 56, 1-18). A number of drug molecules, including, E-4031, cisapride and terfenadine, are all known to prolong the QT interval of the electrocardiogram in man (Fuliki A, *et al.* (1994), Cardiovascular Pharmacol. 23: 374-378; Van Haarst AD et al., (1998) Clin Pharmacol. Ther. 64: 542-546; Honig P.K. et al. (1993) J.A.M.A. 269; 1513-1518).

The launch of new drugs with undetected potentially cardiotoxic side effects could have hazardous consequences and could trigger lethal cardiac dysrhythmias in patients. Late detection of QT prolongation, induced by compounds of pharmacological interest can impede drug discovery and development programs, and consequently have a

profound impact on the outcome of a program. It is desirable, therefore, to test for the potential cardiotoxic side effects of compounds at an early stage of drug development.

According to the invention there is provided an assay that comprises, or consists of, the following steps:

a) incubation of cells expressing ERG or membranes derived from cells expressing ERG or membranes derived from tissue expressing ERG with labelled IKR blocker in assay buffer in the presence or absence of different amounts of a test compound or a mixture of test compounds;
b) determination of specifically bound labelled IKR blocker;
c) calculation of the inhibition of labelled IKR blocker binding by the test compound or mixture of test compounds.

The assay is useful as a preclinical predictive indicator for identification of compounds with a propensity to prolong the QT interval in man. The assay is a competitive binding assay that measures the ability of a test compound or mixture of compounds to displace labelled IKR blocker from the ERG K^+ channel (ether-a-go-go K^+ channel, herein called ERG). The assay can be performed in a high throughput test system. In conjunction with structure-activity relationships (SAR), ligand binding assays using labelled IKR blockers can be used to assist in the design of new drugs devoid of, or with reduced affinity to ERG, in particular human ERG (hERG).

The assay buffer used is particularly important for optimising binding of the IKR blocker or test compound(s) to ERG. It has been found that optimal assay performance is achieved using a Tris based buffer (pH 7.2 to 7.6, preferably pH 7.4 at room temperature) containing potassium (K^+) ions. Potassium ions in the assay buffer may be provided, for example as potassium choride (KCl). The concentration of potassium ions in the assay buffer determines the predictive value of the assay. Assays performed in assay buffer containing from 7.5 to 12.5mM KCl, preferably from 8.5 to 11.5mM KCl, most preferably 10mM KCl are particularly useful to provide an IC_{20} value predictive of onset of QT prolongation.

The assay buffer of the invention preferably comprises or consists of Tris.Cl and KCl. Optionally, $MgCl_2$ may be included in the assay buffer.

The concentration of Tris.Cl in the assay buffer is preferably from 30mM to 100mM Tris.Cl, more preferably from 30mM to 70mM Tris.Cl, yet more preferably from 40mM to 60mM Tris.Cl, further preferably from 45mM to 55 mM Tris.Cl, most preferably 50mM Tris.Cl.

The concentration of KCl in the assay buffer is preferably from 5 to 20mM KCl, more preferably from 6 to 15mM KCl, yet more preferably from 7.5 to 12.5mM KCl, further preferably from 8.5 to 11.5mM KCl, most preferably 10mM KCl.

In a particularly preferred embodiment, the assay buffer comprises or consists of from 30 to 100mM Tris.Cl and from 5 to 20mM KCl, preferably from 30 to 70mM or from 30 to 100mM Tris.Cl and from 6 to 15mM KCl; yet more preferably from 40 to 60mM Tris.Cl and from 7.5 to 12.5mM KCl; further preferably from 45 to 55mM Tris.Cl and from 8.5 to 11.5mM KCl.

It is particularly preferred that the assay buffer comprise or consist of 50mM Tris.Cl and 10mM KCl.

If $MgCl_2$ is included in the assay buffer, the concentration of $MgCl_2$ is preferably from 0.6mM to 2.0mM $MgCl_2$, more preferably from 0.6mM to 1.6mM $MgCl_2$, yet more preferably from 0.8mM to 1.4mM $MgCl_2$, further preferably from 0.9mM to 1.3mM $MgCl_2$, yet further preferably from 1.0mM to 1.2mM $MgCl_2$, most preferably 1.0mM or 1.2mM $MgCl_2$.

In a preferred embodiment the assay buffer used comprises or consists of from 30 to 100mM Tris.Cl, from 5 to 20mM KCl, and from 0.6 to 2.0mM $MgCl_2$; preferably from 30 to 100mM Tris.Cl or from 30 to 70mM Tris.Cl, from 6 to 15mM KCl, and from 0.6 to 1.6mM $MgCl_2$; yet more preferably from 40 to 60mM Tris.Cl, from 7.5 to 12.5mM KCl and from 0.8 to 1.4mM $MgCl_2$; further preferably from 45 to 55mM Tris.Cl, from 8.5 to 11.5mM KCl and from 0.9 to 1.3mM $MgCl_2$ or from 1.0 to 1.2mM $MgCl_2$.

The assay buffer may comprise or consist of 50mM Tris.Cl, 10mM KCl and 1.0mM $MgCl_2$; or 50mM Tris.Cl, 10mM KCl and 1.2mM $MgCl_2$.

It is preferred that the assay buffer be at a pH between 7.2 and 7.6 at room temperature; it is particularly preferred that the assay buffer be at pH 7.4 at room temperature.

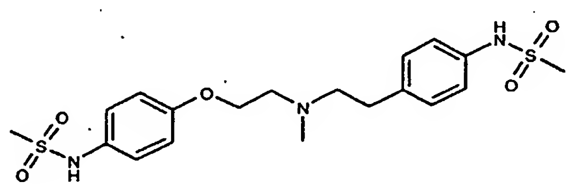
The ERG gene (cDNA) can be from a vertebrate or invertebrate source; for vertebrates the ERG gene may be from a mammalian source (e.g. human, simian, bovine, porcine, canine, rabbit, guinea pig, rat, or mouse) or an invertebrate source such as an insect source (e.g. drosophila). A prokaryotic homologue of mammalian ERG may be used. It is preferred that the ERG gene be mammalian ERG, in particular human ERG (hERG) or canine ERG (cERG).

The ERG gene may be expressed in a mammalian cell line e.g. HEK-293 (Human embryonic kidney) cells, CHO (Chinese hamster ovary) cells; CHL (Chinese hamster lung) cells, COS (monkey) cells; or in an insect cell line e.g. SF9. A baculovirus vector system can be used for expression of ERG in a compatible insect cell line. Alternatively, ERG may be expressed in yeast or bacterial cells. It is preferred that the ERG gene is hERG or cERG and is expressed in either HEK-293, CHO or CHL cells.

The assay may be performed using whole cells expressing ERG or membrane preparations derived from cells expressing ERG, or membrane preparations derived from tissue expressing ERG.

Dofetilide is an IKR blocker (selective inhibitor of the rapid component of the delayed rectifier potassium current), which prolongs the action potential duration and the effective refractory period in a concentration-dependent manner. Clinical studies have demonstrated that dofetilide is effective in treating patients with atrial as well as ventricular arrhythmias. Dofetilide has formula I below.

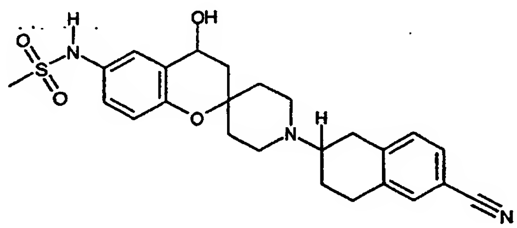
Formula I



Dofetilide is claimed and its preparation is described in European patent EP 0245997.

MK-499 (Merck) is methylsulphonamide antiarrhythmic drug that acts as an IKR blocker. MK-499 has formula II shown below.

Formula II



The IKR blocker used in the assay is labelled with a detectable label, for example a radiolabel or fluorescent tag. In a preferred embodiment of the invention, the labelled IKR blocker used in the assay is labelled dofetilide, preferably radiolabelled dofetilide, most preferably tritiated dofetilide ([^{3}H]-dofetilide). In another embodiment of the invention, the labelled IKR blocker used in the assay is labelled MK-499, preferably radiolabelled MK-499, most preferably tritiated MK-499 ([^{3}H]-MK-499).

Preferred assay formats include the filter binding technique, whereby bound and unbound labelled IKR blocker e.g. labelled dofetilide or labelled MK-499; preferably radiolabelled dofetilide or radiolabelled MK-499; most preferably [^{3}H]-dofetilide or [^{3}H]-MK-499, are separated by filtration. The assay can be performed utilising the scintillation proximity assay (SPA) technique, using radiolabelled IKR blocker e.g. radiolabelled dofetilide or radiolabelled MK-499, preferably [^{3}H]-dofetilide or [^{3}H]-MK-499.

In the filter binding technique, cells expressing ERG or membranes derived from cells expressing ERG or membranes derived from tissue expressing ERG are incubated in assay buffer with labelled IKR blocker e.g. [^{3}H]-dofetilide or [^{3}H]-MK-499, in the presence (test) or absence (control) of the test compound or mixture of test compounds. Incubations are preferably carried out at room temperature for from 60 to 120 minutes, preferably for 90 minutes. Non-specific binding is determined in the

presence of unlabelled IKR blocker, e.g. 10μM dofetilide or 10μM MK-499. Bound labelled IKR blocker is separated from unbound IKR blocker by filtration through filter mats, or onto multiwell filter plates. Filter mats or plates are washed to remove unbound labelled IKR blocker, bound labelled IKR blocker is quantified e.g. for tritiated IKR blocker such as [^{3}H]-dofetilide or [^{3}H]-MK-499 by scintillation spectroscopy using an appropriate counter for radioactivity.

In the scintillation proximity assay™ (SPA) system (Amersham Biosciences), beads are used to bind cells expressing ERG or membranes derived from cells expressing ERG or membranes derived from tissue expressing ERG. A variety of bead types are suitable for use in a SPA assay according to the invention, these include PVT wheat germ agglutinin, yttrium oxide polylysine beads, or yttrium silicate beads (YSi) (Amersham Biosciences) such as YSi polylysine or YSi wheat germ agglutinin. The optimum bead type for use in a SPA assay of the invention depends on the cells or cell membranes used; bead to cell or bead to membrane binding may be assessed to identify the optimum bead type for the cell or cell membrane used. Beads bound to ERG material (whole cell, cell membrane preparation or tissue membrane preparation) are incubated in assay buffer with labelled IKR blocker, e.g. [^{3}H]-dofetilide or [^{3}H]-MK-499 in the presence (test) or absence (control) of the test compound or mixture of test compounds. The ability of the test compound or mixture of test compounds to displace bound radiolabelled IKR blocker is determined by detecting light emissions, for example using standard counters that can be used with SPA technology.

The assay may also include one or more of the steps of: calculation of the concentration of the test compound(s) that gives 20% inhibition of dofetilide binding (IC_{20}), calculation of the concentration of the test compound(s) that gives 50% inhibition of dofetilide binding (IC_{50}), calculation of the compound affinity as Ki or calculation of the compound affinity as pKi.

The IC_{20} values generated from competitive displacement of IKR blocker binding, e.g. [^{3}H]-dofetilide binding, using the assay of the invention are comparable to the free drug concentration associated with QT prolongation in man. Thus the assay can be used to predict the concentration of a compound liable to cause undesirable cardiac side effects.

To assess whether a compound, or mixture of compounds, is likely to prolong the QT interval in the electrocardiogram in man, the following steps are carried out:

a) An assay is carried out according to the invention.

b) An IC_{20} value is obtained; this indicates the real or predicted free drug concentration at which QT prolongation will occur in man;

c) The IC_{20} value is compared with the free drug concentration required for the desired therapeutic effect of the compound or mixture of compounds in vivo.

If the free drug concentration required for the desired therapeutic effect of the compound or mixture of compounds is within 10 to 30 fold of the IC_{20} of the compound or mixture of compounds in the assay, the compound or mixture of compounds is likely to show QT interval prolongation in man.

The assay of the invention is a better predictor of *in vivo* QT prolongation effect of drug molecules than existing assays such as the HERG patch clamp assay.

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Figure 2: Correlation plots comparing p*Ki* values obtained from filter binding and SPA binding assays: (a) correlation between 96 well hERG [^{3}H] dofetilide SPA assay and radioligand binding assay, (b) correlation between 96 well and 384 well hERG [^{3}H] dofetilide SPA assay.

Figure 3: Comparison of inhibition of [^{3}H]-dofetilide binding to hERG, hERG patch clamp, and free drug concentration known to induce QT interval prolongation in man, for (a) E-4031, (b) dofetilide, (c) terfenadine and (d) cisapride.

Figure 4: Comparison of the dofetilide IC_{50} in the dofetilide binding assay carried out in cell membranes from HEK-293 cells transfected with human ERG (hERG (▲)) or with canine ERG (cERG (■))

Figure 5: Comparison of terfenadine IC_{50} in the dofetilide binding assay in cERG or hERG transfected HEK-293 cell membranes

Figure 6: Comparison of E4031 IC_{50} in the dofetilide binding assay in cERG or hERG transfected cell HEK-293 membranes

Figure 7: Mean (n = 2) concentration effect curves for (a) dofetilide and (b) terodiline in tritiated dofetilide SPA assays using assay buffer 50mM Tris Cl, 10mM KCl, at pH7.4.

Examples

Example 1: Preparation of membranes from HEK-293 cells expressing human or canine ERG

An adherent HEK-293 cell line expressing human ERG (Zhou, Z et al (1998) Biophys. J. 74, 230-241) was provided by Dr. Craig January, University of Wisconsin, USA; this cell line was designated the "January" cell line. An alternative adherent HEK-293 cell line, designated cell line 15 (293S-HERG clone 15) was produced by the method described in Zhou, Z et al (1998). Full length cDNA for human ERG was inserted downstream of the CMV promoter in pcDNA3.1 (Invitrogen), the vector also has a SV40 promoter that drives expression of a neomycin resistance gene. The construct was transfected into human embryonic kidney 293S (HEK-293) cells. Stable transformants were selected using G418 (Gibco). Although cell line 15 has slightly lower expression of hERG than the January cell line, it has improved growth characteristics.

Cell line 15 (293S-HERG (Clone 15)) was deposited on 26 June 2002 with the ECACC (CAMR Salisbury, Wiltshire, SP4 OJG, UK) in accordance with the terms of the Budapest Treaty 1977 under deposit accession number 02062678.

Adherent HEK-293 cells expressing human ERG, were grown in MEM Earles medium (Life Technologies) supplemented with 10% foetal calf serum (PAA Laboratories), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 0.4 mg/ml G418 (Life Technologies) and an addition of 1x non-essential amino acids (Life Technologies). The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in T225 cm^3 flasks.

The cells were split 1:3 to 1:5 after reaching 80% confluence using cell dissociation solution (Sigma, cat no: C5914 in 2001) and later seeded into 850 cm^2 CO_2 gassed roller bottles (Corning, cat no: 430849 in 2001) in the absence of G418.

For the preparation of membranes, cells were harvested from the roller bottles by scraping and resuspended in PBS (Life Technologies, cat no: 14190-094 in 2001). All cells were pelleted, washed twice with PBS and snap-frozen on dry ice prior to storage at –80°C until required.

A HEK-293 cell line expressing canine ERG was produced by transient transfection of HEK-293 cells. The complete coding sequence of cERG cDNA (Zehelein et al (2001). Pflugers Archiv. European Journal of Physiology. 442(2): 188 – 191) was provided in the pBluescript® vector (Stratagene) by Professor Zehelein University of Heidelberg, Germany. In the pBluescript construct, the cERG cDNA was flanked by *Bam*HI Sites. Initial experiments indicated poor insertion efficiency for direct insertion of cERG *Bam*HI fragment into the desired vector, pcDNA3.1. To overcome this, an indirect cloning method was devised using the cloning vector pSP73 (Promega). The cERG/pBluescript construct and pSP73 vector were subjected to *Bam*HI digestion, to reduce interference by the presence of pBluescript *Bam*HI fragments in the ligation reaction, the cERG/pBluescript *Bam*HI digested material was also subjected to *Sca*I digestion to cleave pBluescript and ensure more effective separation of the cERG *Bam*HI fragment on agarose gel. The restriction mixtures were subjected to agarose gel electrophoresis, bands containing the cERG and pSP73 *Bam*HI fragments were visualized following staining with ethidium bromide and UV illumination. The cERG and pSP73 bands were excised and eluted from the gel using a QIAgen MinELute Gel extraction kit according to the manufacturers instructions. To prevent religation of the *Bam*HI ends of the pSP73 DNA during the ligation reaction, the plasmid DNA fragments were subjected to CIP treatment using a standard protocol. The cERG *Bam*HI fragments were ligated into the pSP73 *Bam*HI fragments using a standard ligation protocol. After the reaction, the ligation mixture was transformed into cJM109 competent *E. coli* cells using a standard transformation protocol. Transformants were selected by plating on LB agar (Millers) containing ampicillin (50µg/ml) and incubated overnight at 37°C. Overnight cultures of the transformed cells were used to produce mini preparations of cERG/pSP73 DNA using a QIAgen Miniprep kit according to the

manufacturer's instructions. The resulting DNA was subjected to restriction digestion and agarose gel electrophoresis to identify positive clones.

The cERG cDNA was excised from cERG/pSP73 as an *Xho*I (5') *Eco*RI (3') fragment, this fragment was ligated into an *Xho*I/*Eco*RI fragment of the reverse poly linker form of pcDNA3.1, pcDNA3.1(-) *Xho*I/*Eco*RI. In this instance the reverse polylinker form was used because the cERG/pSP73 clone selected contained the reverse orientation of cERG. After ligation into pcDNA3.1(-), the 5' end of cERG was located adjacent to the enhancer-promoter sequence from human cytomegalovirus (CMV). The ligation mixture was transformed into cJM109 competent *E. coli* cells using a standard transformation protocol, transformants were selected via plating onto LB agar (Millers) containing ampicillin (50μg/ml) and incubating overnight at 37°C along with required controls. Colonies picked at random from the cERG/pcDNA3.1(-) plates were inoculated into 5ml of LB media containing ampicillin (50μg/ml) and incubated at 37°C, 200rpm overnight. These overnight cultures were subsequently used to produce mini-preps of DNA using a QIAgen Miniprep Kit. The resulting DNA was subjected to a *Xho*I and *Eco*RI double digestion and analysis on 1% agarose gel. No positive cERG/pcDNA3.1(-) clones were identified because of low insertion efficiency in the ligation reaction coupled with the fact that DNA from only a small number of clones was analysed using the mini prep method. A colony PCR method was thus used to screen a larger number of colonies for positive clones.

The colony PCR protocol permitted rapid detection of cERG/pcDNA3.1(-) clones. Three primers were designed and made for use in the PCR protocol:

Primer 1: 'CERG01' (SEQ ID NO: 1) which hybridises to cERG at nucleotide positions 601-620 of the coding sequence:

5'-ACCACATCCACCAGGCACAG-3'

Primer 2: 'NHE PCDNA3' (SEQ ID NO: 2) which hybridises to pcDNA3.1(-) at nucleotide positions 886-910 (within the multicloning site flanking the *Nhe*1 cloning site):

5'-CCCAAGCTGGCTAGCGTTTAAACGG-3'

Primer 3: 'T7 SP73' (SEQ ID NO: 3) which was used as a control and used against a colony known to produce cERG/pSP73. This hybridised to pSP73 at nucleotide positions 98-121, within the T7 polymerase promoter sequence:

5'-TAATACGACTCACTATAGGGAGA-3'

Ninety-five cJM109 colonies were picked from the LB agar transformation plates and transferred to a sterile deep well 96-well plate containing 1ml/well LB broth supplemented with ampicillin (50µg/ml). As a control, a colony known to contain the cERG/pSP73 plasmid was transferred to the final 96th well containing LB-amp broth. The plate was covered and incubated at 37°C overnight at 200rpm. An aliquot of 70µl of each mini-culture was transferred to a 96-well PCR plate (0.5ml/well) and placed in a Beckman Allegra 6R centrifuge for 2800rpm, room temperature for 10 minutes. The supernatant was discarded and the plate drained for 3 minutes. The PCR reaction mixes were set up and added to the PCR plate containing the bacterial pellets as follows:

	Test wells	Control well cERG/pSP73.
Taqman Gold buffer (X10)	10.0µl	10µl
dNTPs (X10, 2mM/dNTP)	2.0µl	2µl
Taqman Gold Polymerase (5u/µl)	0.5µl	0.5µl
CERG01 (primer 1 - 25µM)	2µl	2µl
NHE PCDNA3 (primer 2 - 25µM)	2µl	
T7 SP73 (primer 3 - 25µM)	-	2µl
Nuclease free water	83.5µl	83.5µl

The bacterial pellet was resuspended in the PCR reaction mixture. The PCR reaction was performed as specified by the manufacturers protocol for the Taqman Gold PCR kit (Applied Biosystems, 1999 edition) thus:

	Temperature	Time	
Step 1 - hot start	95°C	6 minutes	
Step 2 - denaturation	95°C	1 minute	
Step 3 - annealing	60°C	1 minute	
Step 4 - extension	72°C	1 minute	*To step 2 for 35 cycles, then step 5.*

Step 5 - denaturation	95°C	45 secs
Step 6 - annealing	60°C	45 secs
Step 7 - extension	72°C	5 minutes

The PCR products for each well were then separated by electrophoresis on a 1.5% agarose gel using a 100bp DNA ladder marker at 100V for 25 minutes in 1X TAE buffer and visualised using UV light. Putative positive clones were identified and samples from these PCR reaction mixtures were run on a second separate 1.5% agarose gel at 100V for one hour to examine the sizes of the PCR products.

The mini-cultures which gave an amplified a PCR product were each seeded from the original deep-well 96-well plate into sterile tubes with 5ml LB broth containing 50µg/ml ampicillin and incubated at 37°C overnight at 200rpm. The overnight cultures were then used to produce mini-preps of DNA using a QIAgen Miniprep Kit. The resulting DNA was subjected to an *Xho*I and *Eco*RI double digestion to check for the presence of cERG/pcDNA3.1(-). The restriction digest was analysed via a 1% agarose gel run for 1 hour at 100V with 1kb DNA ladder markers (20µl sample loading with 2µl gel loading solution). Further restriction digestion analysis was performed to confirm that the purified plasmids from the transformants were indeed cERG/pcDNA3.1(-).

Untransfected HEK-293 cells were routinely maintained in 50ml Minimum Essential Medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS), 2mM L-glutamine, 1mM sodium pyruvate and 1mM non-essential amino acids. Cells were seeded into 225cm^2 ventilated cap flasks and were maintained in a humidified atmosphere containing 5% CO_2. The HEK-293 cells used in this study were between passage numbers 39-48. Cells were passaged typically every three days in a ratio of 1:3 from a flask of 80-90% confluency; fresh medium was added after washing twice with 10ml PBS and dissociating from the flask using cell dissociation fluid.

The cERG/pcDNA3.1(-) construct was transfected into HEK-293 cells grown to 80-95% confluency in 225cm^2 ventilated flasks using the following method. Endotoxin free cERG/pcDNA3.1(-) DNA (94µg) and Lipofectamine2000 (Gibco BRL) (94µg) were added to 2.25ml of OPTIMEM-I media (Gibco BRL) in sterile 10ml centrifuge tubes; mixing was carried out after incubation at room temperature for five minutes. The

Lipofectamine2000/DNA/OPTIMEM-I mix was then incubated at room temperature for twenty minutes before the addition of a further 10.5ml OPTIMEM-I. HEK-293 cells were washed with 10ml PBS and the Lipofectamine2000/DNA/OPTIMEM-I mixture added and incubated for 3.5 hours at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, 50ml of MEM (supplemented with 10% (v/v) FCS, 2mM L-glutamine, 1mM sodium pyruvate and 1mM non-essential amino acids) was added. The HEK-293 cells were incubated for 24 hours at 37°C. Transfected cells were harvested after 24 hours by washing with PBS, scraping the cells into 10ml PBS and centrifuging at 1000rpm for 5 minutes at room temperature. The resulting cERG/pcDNA3.1(-) transfected HEK-293 cell pellet was stored at -80°C until required.

Preparation of membranes from HEK-293 cells expressing human or canine ERG

Cell membrane fractions were prepared from frozen aliquots of cells. All procedures were carried out at 4°C unless otherwise stated. Frozen aliquots of cells were thawed at room temperature and resuspended in assay buffer (e.g. 50mM Tris.Cl, 10mM KCl, 1 to 1.2mM $MgCl_2$, pH7.4, or 50mM Tris.Cl, 10mM KCl, pH7.4). The cells were then disrupted by homogenisation in an Omni LabTek homogeniser at 20,000 rpm for 30 seconds. The homogenate was centrifuged for 20 minutes at 48,000xg (4°C, Sorvall RC5B centrifuge) and the supernatant removed. The resulting pellets were resuspended in assay buffer and homogenised as above for 10 seconds. The pellets were collected by centrifugation and the final pellet resuspended in assay buffer. Protein content was determined using a Coomassie Blue based protein assay kit. Aliquots were stored at -80°C until needed; when stored in these conditions, the binding ability of the cell membrane fractions proved to be stable for at least 4 months.

Example 2: Filter binding assay with [^{3}H]-dofetilide

[^{3}H]-dofetilide (80-83 Ci/mmol) was synthesized by catalytic tritiation (a custom service provided, for example, by Amersham Life Science). However, other detectable labels known to the skilled person can be used instead of ^{3}H, e.g. fluorescent tags, other radiolabels, antibodies etc.

On the day of the assay, test compounds were dissolved at 1 mM in 50% DMSO or 100% DMSO, and then diluted to the desired concentrations (e.g. up to 100µM, or up to

the boundaries of solubility for the compound) in assay buffer. The final DMSO concentration in assay incubations is preferably 1.0 to 1.5% or less for optimal assay conditions.

Incubations included membrane homogenate at 50µg/ml in assay buffer (50 mM Tris.Cl, 10mM KCl, 1.0mM to 1.2mM $MgCl_2$, pH7.4) unless otherwise indicated, [^{3}H]-dofetilide (4 to 7nM) and test compound or mixture of test compounds or control vehicle. Filtration assays were incubated at room temperature for 90 minutes. Non-specific binding was determined in the presence of 10 µM dofetilide and was usually less than 15 % of total binding. Bound ligand was separated from free ligand by rapid filtration through GF/B glass fibre filter mats using, for example, a Brandel cell harvester, or onto GF/B Unifilter 96-well filter plates (Packard) using a Packard Filtermate 96 harvester. Filter mats and plates were pre-soaked in 5% PEI (w/v) for 60 minutes and washed after harvesting with 3 x 1 ml washes of ice-cold assay buffer. Unifilter plates were air dried for a minimum of 1.5 hours at 37°C prior to the addition of Microscint-0 (Packard). Bound [^{3}H]-dofetilide was determined by liquid scintillation spectroscopy using an appropriate counter, for example in a Packard TopCount Scintillation Counter (NXT Counter) or Wallac Counter (Trilux) for Unifilter plates and in a Wallac Big Spot Counter when filter mats were used.

In each experiment, triplicate assays were routinely performed and the data were averaged. Specific binding was analysed by nonlinear regression fit using GraphPad Prism software (GraphPad, San Diego). IC_{50} values were derived from a 4 parameter logistic fit using PRISM and converted to Ki values by use of the Cheng & Prusoff equation; IC_{20} values were extrapolated from the graph.

Example 3: Scintillation proximity assay

The scintillation proximity assay (SPA) was carried out in assay buffer consisting of 50mM Tris.Cl, 10mM KCl, 1.0mM to 1.2mM $MgCl_2$, pH7.4, or using assay buffer consisting of 50mM Tris base, 10mM KCl, pH7.4. Bead to membrane binding was assessed to determine the optimum bead type for the cell line used. YSi wheatgerm agglutinin beads were used with cell membranes derived from the January HEK-293 hERG expressing cell line; YSi polylysine beads were used in studies using membranes

derived from Cell Line 15 (HEK-293 hERG expressing cell line). Conditions were optimised with respect to bead and cell membrane homogenate concentration, prior to characterising ERG pharmacology. The incubations (200 µl total per well for 96 well plates and 60 µl total per well for 384 well plates) included 25 µg of cell membrane homogenate per mg of bead. The membrane homogenate was precoupled with the YSi Wheatgerm Agglutinin or YSi polylysine bead suspension at 4°C on a roller shaker for approximately 2 hours. For competition binding assays, membrane homogenate bead suspension was incubated in white clear bottom 96 or 384 well plates with 5nM [^{3}H]-dofetilide in the absence and presence of competitor i.e. the test compound or mixture of test compounds. The plates were incubated at room temperature and shaken for approximately 1 hour. Beads were allowed to settle for a minimum of 30 minutes before plates were counted for retained radioactivity on a TopCount NXT scintillation counter. Nonspecific binding i.e. background count, was determined by the addition of 10µM dofetilide. Background counts were usually less than 15% of the total binding. For saturation studies, specific binding of [^{3}H]-dofetilide was determined over a range of concentrations (5 to 500nM) in the absence or presence of cold (i.e. unlabelled) 10µM dofetilide.

Example 4: Assay optimisation

a) Effect of Hepes- and Tris-based buffers on dofetilide binding

To optimise the specific binding of dofetilide to homogenates of cell membranes containing ERG, the interaction of [^{3}H]-dofetilide with the cell membrane preparation was examined in the presence of Hepes-based buffer (25mM Hepes, 135mM NaCl, 5mM KCl, 1mM $MgSO_4$, 50mM $CaCl_2$, pH7.4) and Tris-based buffer (50mM Tris.Cl, 10mM KCl, 1mM or 1.2mM $MgCl_2$). Comparison of the specific binding in these buffers revealed that percentage specific binding was similar in both Tris-based and Hepes-based buffers. However, as shown in Table 1, specific counts were twice as high in the presence of Tris-based buffer compared to those detected in Hepes-based buffer.

Table 1. Comparative effects of Tris-based and Hepes-based buffers on [^{3}H]-dofetilide binding to cell membrane homogenate expressing hERG.

Buffer	25mM HEPES free acid 135mM NaCl, 5mM KCl 1mM $MgSO_4$, 50 µM $CaCl_2$ pH 7.4 at room temp	50mM Tris 10mM KCl and 1.0 or 1.2mM $MgCl_2$ pH 7.4 at room temp
Total Binding (ccpm)	8510 ± 669	19627 ± 1189
Non-specific Binding (ccpm)	321 ± 27	315 ± 23
Specific Binding (ccpm)	8189	19312
% Specific Binding	96	98

Total and non-specific binding data represent arithmetic mean ± standard error mean of 14 individual wells per buffer split over two assays, performed at a protein concentration of 75µg/ml and a mean [^{3}H]-dofetilide concentration of 6.7nM. Incubation was carried out for 60 minutes at room temperature. ccpm=corrected counts per minute.

So that the maximum specific binding window could be achieved, the assay buffer used in Examples 1 to 8 was the Tris-based incubation buffer (50mM Tris.Cl, 10mM KCl, 1mM $MgCl_2$). Additionally, experiments were performed to optimise the cell membrane protein concentration and bead concentration for filter and SPA binding assays.

b) Saturation binding

Time courses were performed to determine optimal incubation time for binding activities. Incubation times were similar for both filter binding and SPA assays. The filter binding assay reached equilibrium in 90 minutes, SPA required 60 minutes. [^{3}H]-dofetilide binding to ERG in both filter binding and scintillation proximity assays was saturable with a K_D of 5.08 ± 1.0nM for filter binding and K_D values of 8.9 ±0.6nM and 9.1 ±1.8nM for 96 and 384 format scintillation proximity assays respectively (Figure 1a-c, with Fig. 1a showing the results of the filter binding assay, Fig. 1b the results of the SPA in 96-well format, and Fig. 1c showing the results of the SPA in 384 well format). Non-linear curve fitting of this data indicated that binding was to a single site. A B_{max} of 7.4 ± 0.7pmol/mg protein for [^{3}H]-dofetilide was obtained from filter binding (Figure 1). As scintillation proximity assays do not give an accurate determination of dpm (disintegrations per minute) values, a B_{max} is not quoted for SPA.

c) **Comparison of SPA and filter binding techniques**

A comparison of SPA and filter binding techniques revealed excellent concordance of results. Affinity values displayed excellent correlation between the two assay types and the rank order of compound affinity is identical, as is shown in Figure 2 (correlation plots comparing p*Ki* values obtained from filter binding and SPA binding assays).

d) **Competitive binding studies**

A range of compounds, including hERG blockers known to prolong the QT interval in man, was examined for competitive displacement of [^{3}H]-dofetilide. E4031, dofetilide, terfenadine, and cisapride produced complete inhibition of specific binding with a range of calculated affinity values that are summarised in Table 2.

Table 2. Affinity values for compounds tested against [^{3}H]-dofetilide filter and SPA binding assays to HERG.

Compound	Filter binding pK_i	SPA 96 pK_i	SPA 384 pK_i
Dofetilide	8.22 ± 0.04	8.05 ± 0.54	8.26 ± 0.12
E4031	7.82 ± 0.03	7.81 ± 0.05	7.89 ± 0.11
Terfenadine	7.53 ± 0.09	7.75 ± 0.07	7.72 ± 0.41
Cisapride	7.34 ± 0.05	7.15 ± 0.04	7.55 ± 0.22
Glibenclamide	< 5	< 5	< 5
D-Sotalol	< 5	< 5	< 5

Data expressed as p*Ki* values (the negative logarithm of molar concentration of competing ligand to displace 50% of 5nM [^{3}H]-dofetilide binding). Data are the mean of at least n = 3 experiments.

Example 5: Prediction of QT interval prolongation effect of compounds in man

The IC_{20} values generated from competitive displacement of [^{3}H]-dofetilide binding using the assay of the invention are comparable to the free drug concentration associated with QT prolongation in man as is shown in Figure 3 for a range of compounds, including E-4031 (Figure 3a), dofetilide (Figure 3b), terfenadine (Figure 3c) and cisapride (Figure 3d). For each compound, the inhibition of dofetilide binding in the binding assay (filter binding technique), and in a hERG patch clamp assay is compared with the concentration of free drug associated with QT interval prolongation in man (Fuliki A, *et al.* (1994) Cardiovascular Pharmacol, 23: 374-378; Van Haarst AD et al. (1998) Clin Pharmacol. Ther. 64: 542-546; Honig PK, et al. (1993) J.A.M.A. 269: 1513-1518).

The ERG patch clamp assay provides a measure of the current through the ERG channel and indicates the number of ion channels present in a cell. However, due to the phenomena of state dependent block observed in patch clamp studies (Walker, B.D. et al (1999) British J. Pharmacol 128, 444-450) exhibited by a number of known hERG blockers with the propensity to prolong the QT interval *in vivo*, the ligand binding assay provides a better predictor of *in vivo* QT prolongation effect of a drug than the hERG patch clamp technique (Figure 3d).

To assess whether a compound or mixture of compounds is likely to prolong the QT interval in the electrocardiogram in man, the following steps are carried out:

a) A binding assay is carried out according to the invention, for example as as described in Example 2 or Example 3, to test the affinity of the compound or mixture of compounds for ERG, preferably hERG or cERG;

b) The IC_{20} is obtained, e.g. as described at the end of Example 2; the IC_{20} being the real or predicted free drug concentration at which QT prolongation occurs in man;

c) The IC_{20} value is compared with the free drug concentration required for the desired therapeutic effect of the compound in vivo.

If the free drug concentration required for the desired therapeutic effect of the compound is within 10 to 30 fold of the IC_{20} of the compound in the assay of the invention, the compound is highly likely to cause QT interval prolongation in man.

Example 6: Comparison of dofetilide binding assay carried out HEK-293 cells transfected with cERG or hERG.

The dofetilide binding assay was carried out as described Example 2 using HEK 293 cells transfected with either human ERG or canine ERG. The results are shown in figure 4, from which it can be seen that the IC_{50} for dofetilide is similar for canine and human ERG, being 13.9nM and 15.6nM respectively. IC_{20} values for dofetilide were 1.92nM and 2.15nM for canine and human ERG, respectively.

Example 7: Comparison of terfenadine competition assay using HEK-293 cells transfected with cERG or hERG

The dofetilide binding assay was carried out using terfenadine as the test compound. Transiently transfected cERG HEK-293 cell membranes (200µg/well), or stable hERG HEK-293 cell membranes (100µg/well) were incubated with twelve different concentrations of terfenadine and 5nM [^{3}H]-dofetilide for 90 minutes at room temperature. Total and non-specific binding were measured by incubating with 10% DMSO and 10µM unlabelled dofetilide to a total assay volume of 200µl. The membranes were harvested by filtration with a Packard Unifilter cell harvester and radioactivity (cpm) was measured. Two saturation experiments were carried out each for cERG and hERG expressing cell membrane samples. Each experiment was carried out in triplicate. Figure 5 shows the mean values of the experiments for each cell type (cERG or hERG transfected) and indicates that the IC_{50} for terfenadine is similar for cERG and hERG, being 77.2nM and 88.9nM respectively. IC_{20} values for terfenadine were 10.7nM and 12.3nM for canine and human ERG, respectively.

Example 8: Comparison of E4031 competition assay in HEK-293 cells transfected with cERG or hERG

The dofetilide binding assay was carried out using E4031 as the test compound. Transiently transfected cERG HEK-293 cell membranes (200µg/well) or stable hERG HEK-293 cell membranes (100µg/well) were incubated with twelve different concentrations of E4031 and 5nM [^{3}H]-dofetilide for 90 minutes at room temperature. Total and non-specific binding values were measured by incubation with 10% DMSO and 10µM unlabelled dofetilide in a total assay volume of 200µl. The membranes were harvested by filtration with a Packard Unifilter cell harvester and radioactivity (cpm) was measured. Two saturation experiments were carried out each for cERG and hERG expressing cell membrane samples. Each experiment was carried out in triplicate. Figure 6 shows the mean values of the experiments for each cell membrane type (cERG or hERG transfected) and indicates that the IC_{50} for E4031 is similar for cERG and hERG, being 27.3 nM and 35.4 nM respectively. IC_{20} values for E4031 were 3.8 nM and 4.9 nM for canine and human ERG, respectively.

When IC_{50} (or IC_{20}) values are compared for the compounds tested, they were found to be very similar for cERG and hERG. This indicates that either hERG or cERG can be used in the assay of the invention to predict the onset of QT prolongation in man.

Example 9: Further assay optimisation studies

To further optimise the assay for specific binding of dofetilide to homogenates of cell membrane containing hERG, the interaction of [^{3}H]-dofetilide with cell membrane preparations was examined in the SPA assay format using a Tris based buffer containing either KCl or $MgCl_2$. SPA assays were performed according to example 3 in 50mM Tris.Cl, 10mM KCl at pH7.4 or in 50mMTris.Cl, 1mM $MgCl_2$ at pH 7.4 as the assay buffer. Assays were performed using dofetilide or terodiline as the test compound. Comparison of specific binding detected in these buffer conditions revealed that specific binding was not observed when the assay buffer used was 50mMTris.Cl, 1mM $MgCl_2$ at pH 7.4; specific binding was observed in assay buffer consisting of 50mM Tris.Cl, 10mM KCl at pH7.4. For the assays carried out in 50mM Tris.Cl, 10mM KCl at pH7.4 as the assay buffer the IC_{50} and IC_{20} values were generated for each test

compound. The mean IC_{50} value for dofetilide was 8.69± 0.45nM, the mean IC_{50} value for terodiline was 1.87 ± 0.00 μM. The mean IC_{20} value for dofetilide was 1.2nM, the mean IC_{20} value for terodiline was 0.248μM.

Sequence Listing Information

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Claims

1. An assay comprising or consisting of the following steps:
(a) incubation of cells expressing ERG, or membranes derived from cells expressing ERG, or membranes derived from tissue expressing ERG, with labelled IKR blocker in assay buffer in the presence or absence of a test compound or a mixture of test compounds;
(b) determination of specifically bound labelled IKR blocker;
(c) calculation of the inhibition of labelled IKR blocker binding by the test compound or mixture of test compounds.
2. An assay according to claim 1, wherein the assay buffer is a Tris based buffer containing KCl.
3. An assay according to claim 2, wherein the assay buffer comprises or consists of from 30 to 100mM Tris.Cl, from 5 to 20mM KCl, and optionally from 0.6 to 2.0mM $MgCl_2$.
4. An assay according to claim 2, wherein the assay buffer comprises or consists of from 30 to 70mM Tris.Cl, from 6 to 15mM KCl, and optionally from 0.6 to 1.6mM $MgCl_2$.
5. An assay according to claim 2, wherein the assay buffer comprises or consists of from 40 to 60mM Tris.Cl, from 7.5 to 12.5mM KCl and optionally from 0.8 to 1.4mM $MgCl_2$.
6. An assay according to claim 2, wherein the assay buffer comprises or consists of from 45 to 55mM Tris.Cl, from 8.5 to 11.5mM KCl and optionally from 0.9 to 1.3 mM $MgCl_2$ or from 1.0 to 1.2mM $MgCl_2$.
7. An assay according to claim 2, wherein the assay buffer comprises or consists of 50mM Tris and 10mM KCl.
8. An assay according to claim 2, wherein the assay buffer comprises or consists of 50mM Tris, 10mM KCl and 1.0mM $MgCl_2$; or 50mM Tris, 10mM KCl and 1.2 mM $MgCl_2$.
9. An assay according to any one of the preceding claims wherein the assay buffer is at a pH between pH7.2 and pH7.6 at room temperature.
10. An assay according to claim 9, wherein the assay buffer is at pH7.4.
11. An assay according to any one of the preceding claims wherein the ERG is human ERG.

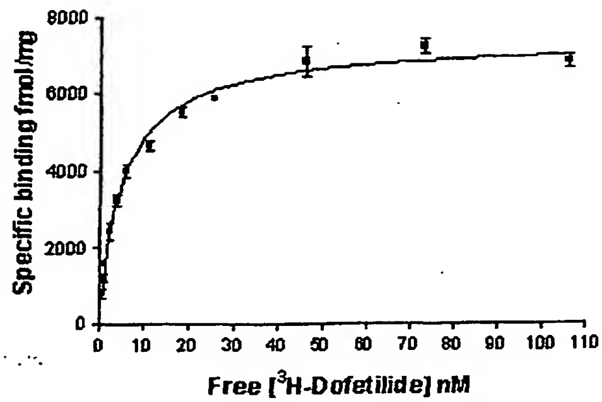
12. An assay according to any one of the preceding claims, wherein the labelled IKR blocker is labelled dofetilide or labelled MK-499.
13. An assay according to claim 12, wherein the labelled dofetilide or labelled MK-499 is radiolabelled.
14. An assay according to claim 13, wherein the radiolabel is tritium (^{3}H).
15. An assay according to any one of the preceding claims having the following additional step(s):

    (d) calculation of the IC_{20} for the test compound or mixture of test compounds, and optionally,

    (e) comparison of the IC_{20} value of the test compound or mixture of test compounds with the concentration required for the desired therapeutic effect of the compound in vivo.
16. An assay according to any one of the preceding claims wherein the assay is performed as a filter binding assay.
17. An assay according to any one of claims 1 to 15 wherein the assay is performed as a scintillation proximity assay.

Figure1

(a)


Specific binding fmol/mg
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6000
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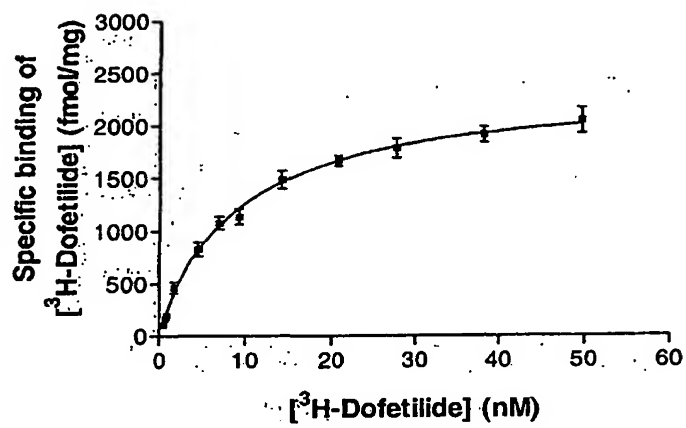
Free [3H-Dofetilide] nM

(b)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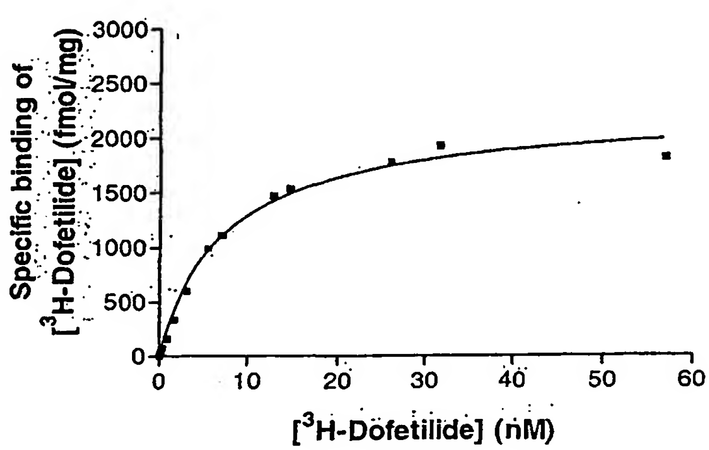
(c)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2000
1500
1000
500
0
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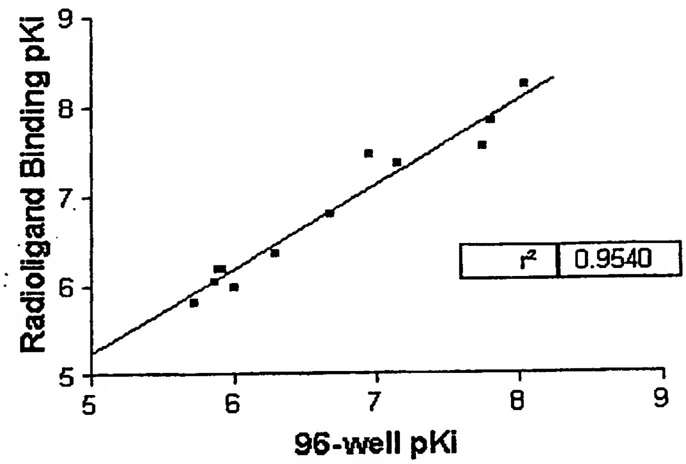
Figure 2

(a)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r²
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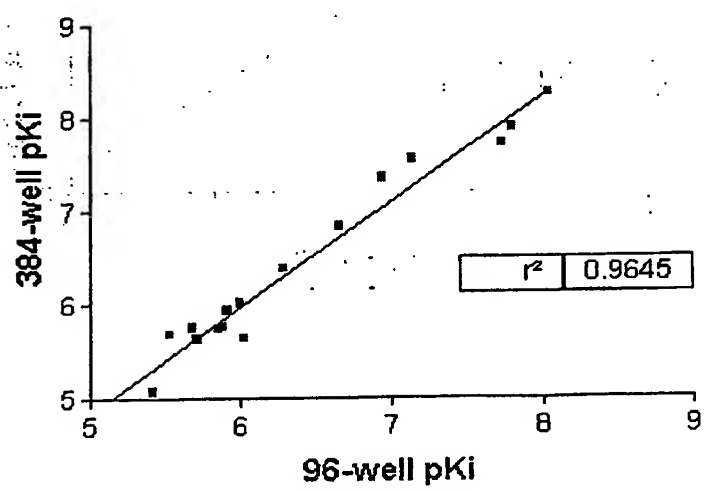
5
6
7
8
9

(b)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r²
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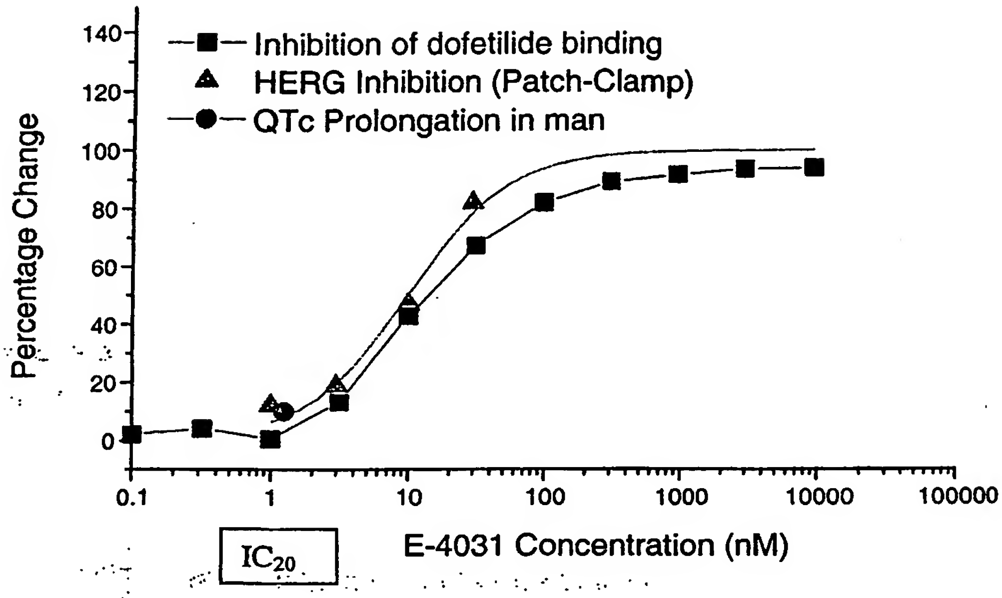
5
6
7
8
9

Figure 3

(a)


Inhibition of dofetilide binding
HERG Inhibition (Patch-Clamp)
QTc Prolongation in man
Percentage Change
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120
100
80
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1
10
100
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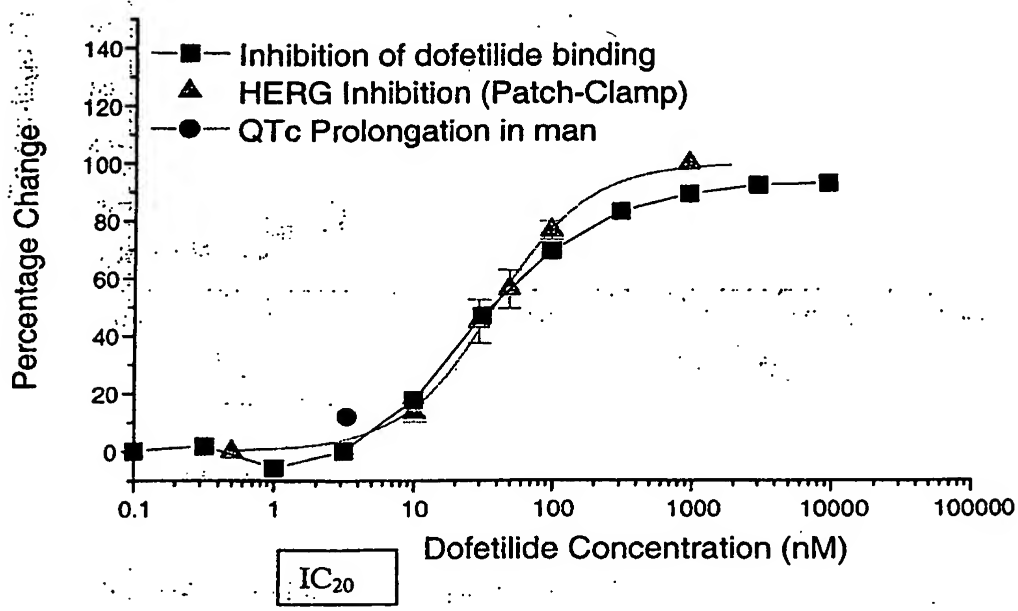
10000
100000
IC20
E-4031 Concentration (nM)

(b)


Inhibition of dofetilide binding
HERG Inhibition (Patch-Clamp)
QTc Prolongation in man
Percentage Change
140
120
100
80
60
40
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1
10
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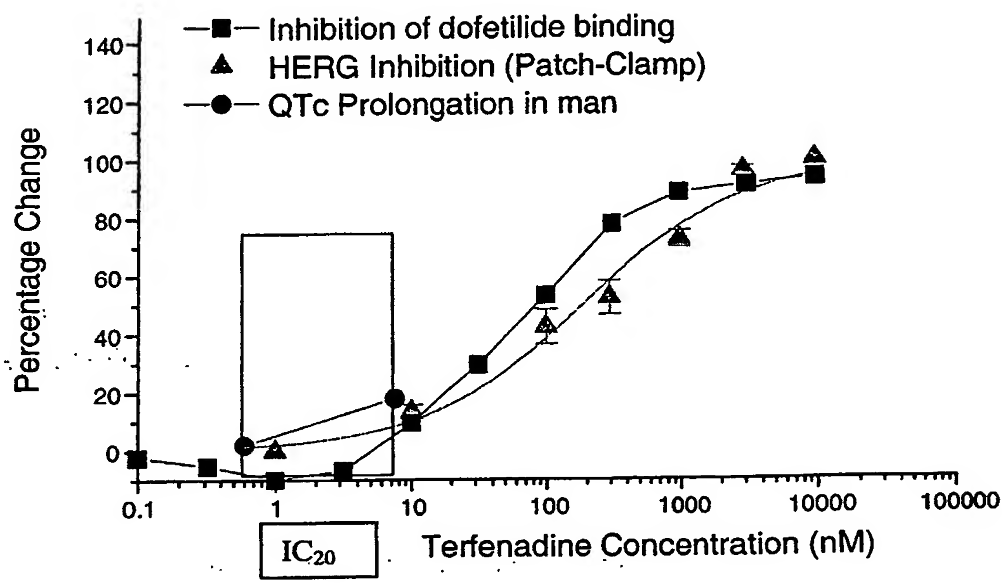
1000
10000
100000
IC20
Dofetilide Concentration (nM)

Figure 3 continued

(c)



(d)

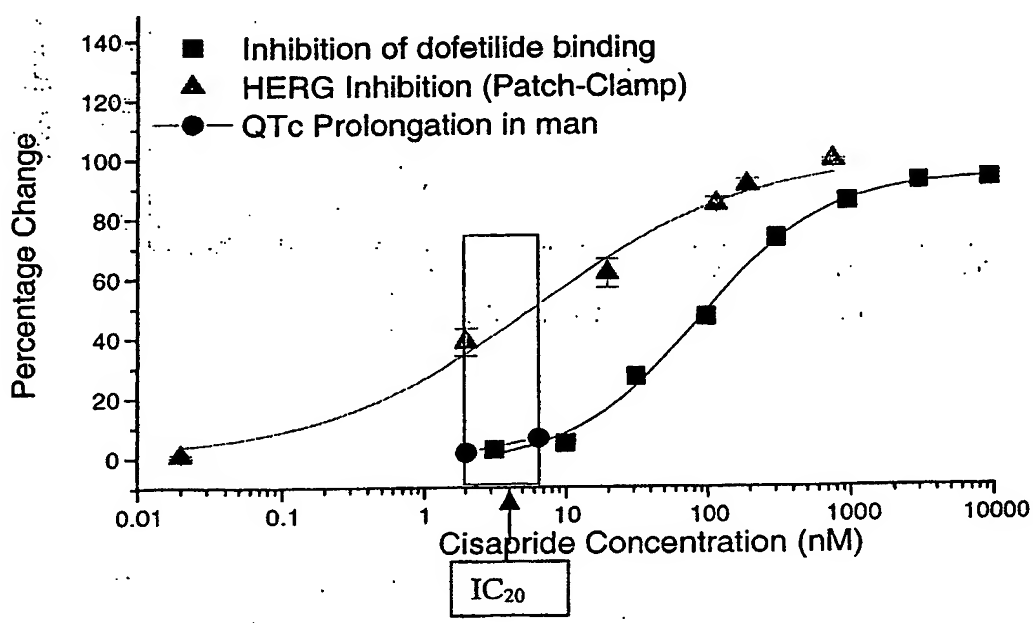
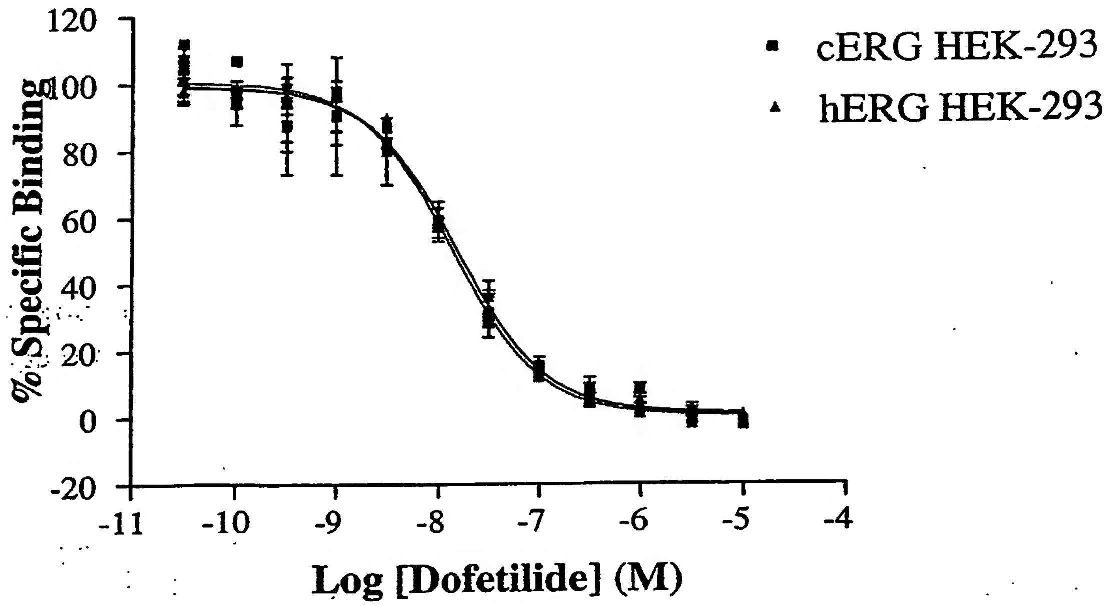


Figure 4


120
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80
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0
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% Specific Binding
-11
-10
-9
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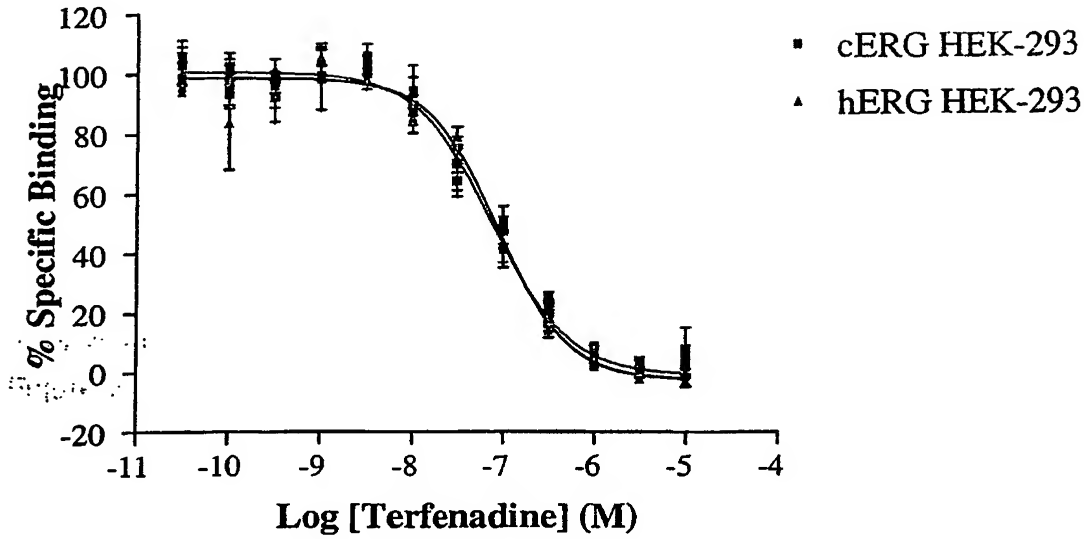
-4
Log [Dofetilide] (M)
cERG HEK-293
hERG HEK-293

Figure 5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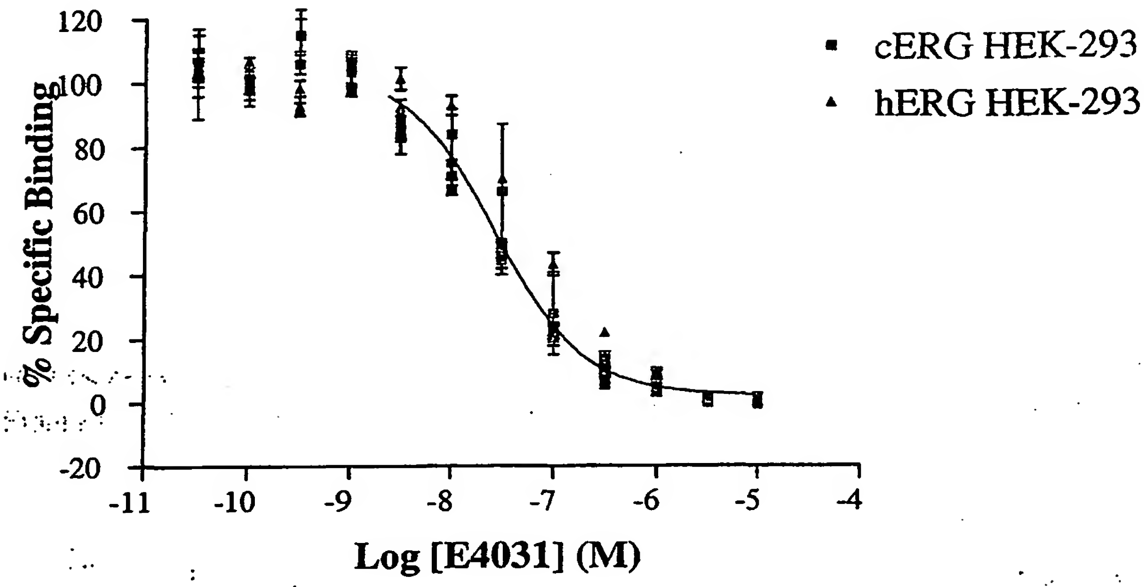
Log [Terfenadine] (M)
cERG HEK-293
hERG HEK-293

Figure 6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0
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% Specific Binding
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-10
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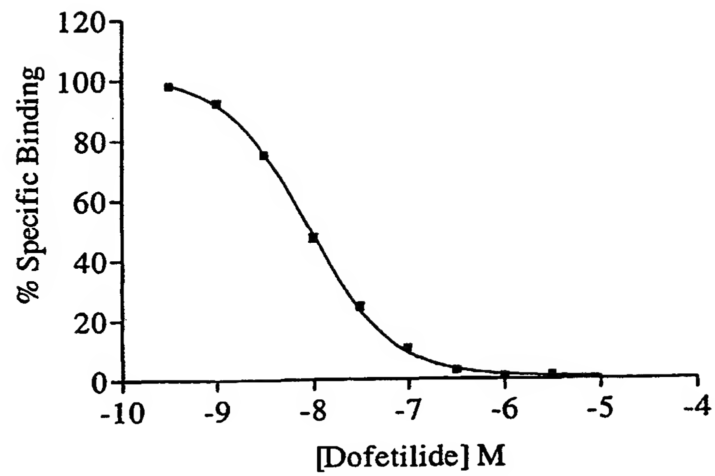
Log [E4031] (M)
cERG HEK-293
hERG HEK-293

Figure 7

(a)


120
100
80
60
40
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% Specific Binding
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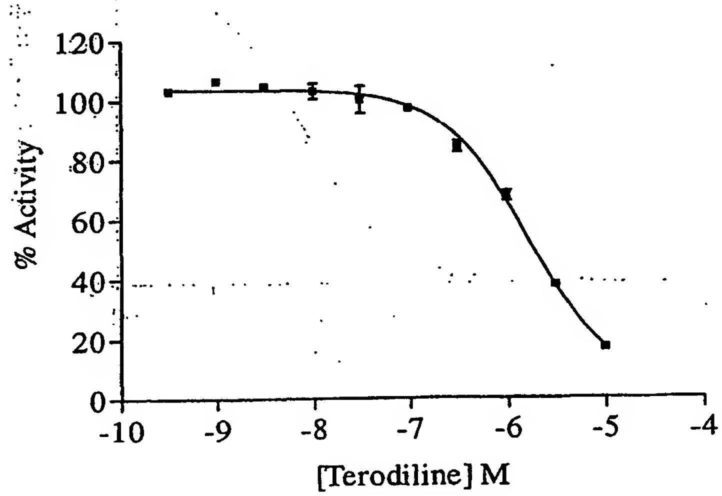
-9
-8
-7
-6
-5
-4
[Dofetilide] M

(b)


120
100
80
60
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% Activity
-10
-9
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(71) Applicant (*for GB only*): **PFIZER LIMITED** [GB/GB]; Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

(71) Applicant (*for all designated States except GB, US*): **PFIZER INC.** [US/US]; 235 East 42nd Street, New York, NY 10017 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **GREENGRASS, Pamela, May** [GB/GB]; Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB). **STEWART, Michael** [GB/GB]; Pfizer Limited, U.K. Patent Department, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB). **WOOD, Claire, Margaret** [GB/GB]; Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

(74) Agents: **HAYLES, James, R.** et al.; Pfizer Limited, Ramsgate Road, Sandwich, Kent CT13 9NJ (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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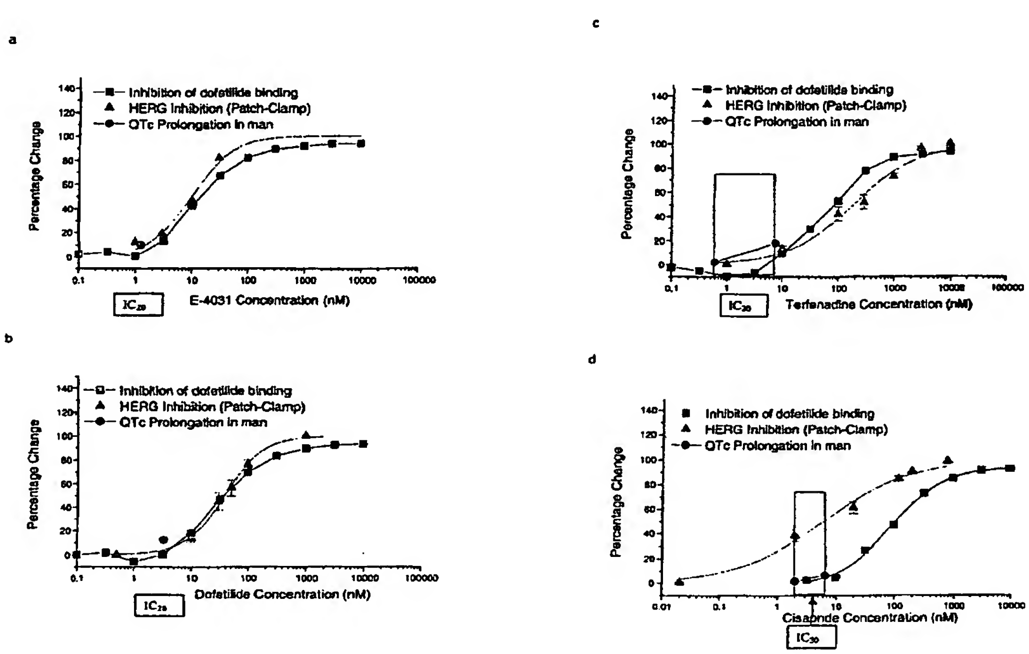
[Continued on next page]

(54) Title: AFFINITY-ASSAY FOR THE HUMAN ERG POTASSIUM CHANNEL



(57) **Abstract:** The invention relates to an assay to establish the affinity of compounds at the "ether-a-go-go" (ERG) potassium (K+) channel, in particular the human ERG (hERG) potassium channel, using a labelled inwardly rectifying potassium channel (IKR) blocker. This assay is useful to identify compounds with undesirable effects on cardiac repolarisation in man, in particular the propensity to prolong the QT interval in the electrocardiogram.

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TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 02/03618

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/566 G01N33/50 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	NETZER RAINER ET AL: "Screening lead compounds for QT interval prolongation" DRUG DISCOVERY TODAY, ELSEVIER SCIENCE LTD, GB, vol. 6, no. 2, January 2001 (2001-01), pages 78-84, XP002198162 ISSN: 1359-6446 abstract page 78, right-hand column, paragraph 2 -page 79, left-hand column, line 3 page 79, right-hand column, last paragraph -page 80, left-hand column, paragraph 1 page 81, right-hand column, last paragraph -page 82, left-hand column, paragraph 1; table 3 --- -/--	1,9-17 2-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2003	13/10/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luis Alves, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FINLAYSON KEITH ET AL: "dofetilide binding in SHSY5Y and HEK293 cells expressing a HERG-like K+ channel?" EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 412, no. 3, 2001, pages 203-212, XP001155254 ISSN: 0014-2999 abstract page 204, left-hand column, last paragraph -right-hand column, paragraph 1 page 206, left-hand column, last paragraph -page 207, left-hand column, paragraph 1	2-8
P,X	FINLAYSON KEITH ET AL: "'3H!Dofetilide binding to HERG transfected membranes: A potential high throughput preclinical screen" EUROPEAN JOURNAL OF PHARMACOLOGY, AMSTERDAM, NL, vol. 430, no. 1, 26 October 2001 (2001-10-26), pages 147-148, XP002198164 ISSN: 0014-2999	1,9-17
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P,X	WO 02 05860 A (CONNOLLY THOMAS M ;KOSTURA MATTHEW J (US); DEAN DENNIS C (US); BUT) 24 January 2002 (2002-01-24) example 3	1-17
A	CAVERO I ET AL: "DRUGS THAT PROLONG QT INTERVAL AS AN UNWANTED EFFECT: ASSESSING THEIR LIKELIHOOD OF INDUCING HAZARDOUS CARDIAC DYSRHYTHMIAS" EXPERT OPINION ON PHARMACOTHERAPY, ASHLEY, LONDON,, GB, vol. 1, no. 5, July 2000 (2000-07), pages 947-973, XP008022068 ISSN: 1465-6566 abstract page 964, right-hand column, last paragraph -page 966, left-hand column, paragraph 3	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 02/03618

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WO 0205860 A	24-01-2002	AU 7786301 A	30-01-2002
		CA 2415295 A1	24-01-2002
		EP 1311300 A1	21-05-2003
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